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Induction of apoptosis by esculetin in human leukemia cells

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Abstract

Esculetin, a coumarin compound, has been shown to exhibit antioxidant and anti-inflammatory effects. In the present study, esculetin was found to inhibit the survival of human promyelocytic leukemia HL-60 cells in a concentration-dependent and time-dependent manner. HL-60 cells underwent internucleosomal DNA fragmentation and morphological changes characteristic of apoptosis after a 24-h treatment with esculetin (100 μM). Flow cytometric analysis showed that the hypodiploid nuclei of HL-60 cells were increased to 40.93% after a 36-h treatment with esculetin (100 μM). Further investigation showed that esculetin induced the release of cytochrome *c* from mitochondria into cytosol in a time-dependent and concentration-dependent manner. Moreover, esculetin application reduced Bcl-2 protein expression to 58% after 9 h as compared with that time at 0. Cysteine protease 32 kDa proenzyme (CPP32), a caspase 3, was activated and its substrate, poly (adenosine diphosphate-ribose) polymerase, was cleaved after a 24-h treatment of HL-60 cells with esculetin. These data suggest that esculetin induces apoptosis in human leukemia cells by increasing cytosolic translocation of cytochrome *c* and activation of CPP32.

Keywords: Apoptosis; Bcl-2; CPP32; Cytochrome *c*; Esculetin; Leukemia

1. Introduction

Esculetin (Fig. 1) is a coumarin derivative contained in many plants, such as *Artemisia capillaris* (*Compositae*), the leaves of *Citrus limonia* (*Rutaceae*) (Chang et al., 1996) and *Ceratostigma willmottianum* (Yue and Xu, 1997), which are used as folk medicines. It has multiple biological activities including the inhibition of xanthine oxidase activity (Egan et al., 1990), platelet aggregation (Okada et al., 1995) and *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in rats (Matsunaga et al., 1998). It also decreases the activity of ferric soybean lipoxygenase 1 (Kemal et al., 1987) and 5-lipoxygenase (Neichi et al., 1983). In addition, esculetin has antioxidant activity (Paya et al., 1992; Lin et al., 2000), an inhibitory effect on the growth of human breast cancer cells (Noguchi et al., 1995), and a synergistic effect with retinoic acid on the differentiation of human leukemia cells (Hofmanova et al., 1998). However, the antitumor mechanisms of esculetin are not well understood. In this study, we investigated the antitumor potential and mode of action of esculetin on human leukemia HL-60 cells.

Recently, considerable attention has been focused on the sequence of events referred to as programmed cell death, or apoptosis, and the role of this process in mediating the lethal effects of diverse antineoplastic agents in leukemia cells (Kaufman, 1989). Apoptosis is a highly regulated process that involves the activation of a cascade of molecular events leading to cell death that is characterized by cell shrinkage, membrane blebbing, chromatin condensation and formation of a DNA ladder with multiple fragments of 180–200 bp caused by internucleosomal DNA cleavage (Steller, 1995). Cells undergoing apoptosis were found to have an elevation of cytochrome *c* in the cytosol and a corresponding decrease in the mitochondria (Yang et al., 1997). After the release of mitochondrial cytochrome *c*, cysteine protease 32 kDa proenzyme (CPP32), a caspase 3, is activated by proteolytic cleavage into a 20 and 10 kDa active heterodimer (Nicholson et al., 1995). Activated CPP32 is responsible for the proteolytic degradation of poly (ADP-ribose) polymerase, which occurs at the onset of apoptosis (Lazebnick et al., 1994; Tewari et al., 1995).

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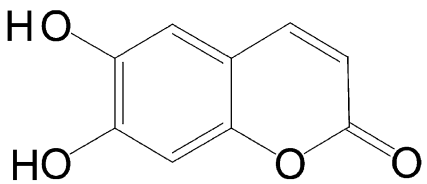

Fig. 1. Structure of esculetin.

Many studies have demonstrated that polyphenols in medical and edible plants have various pharmacological activities, such as anti-inflammatory/antioxidant and anti-carcinogenic activities (Huang and Ferraro, 1992; Newmark, 1992). Recently, scientists reported that natural antioxidants, such as quercetin (Wei et al., 1994), gallic acid (Inoue et al., 1994) and tea polyphenols (Zhao et al., 1997), inhibited the growth of cancer cells through the induction of apoptosis. Esculetin, a plant phenol exhibiting antioxidant and anti-inflammatory properties, was found to suppress cancer cell proliferation (Noguchi et al., 1995; Hofmanova et al., 1996). Here, we present evidence demonstrating that esculetin induces apoptosis of human leukemia cells and that this effect is mediated by an increased cytosolic translocation of cytochrome *c* and activation of CPP32.

2. Materials and methods

2.1. Chemicals

Phosphate-buffered saline (PBS) and RPMI medium 1640 were purchased from GIBCO, (BRL Life Technologies, Grand Island, NY). Mouse monoclonal antibodies against cytochrome *c*, CPP32, PARP and Bcl-2 were purchased from Transduction Laboratories. The enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science (Amersham, UK). The protein assay kit was from Bio-Rad Laboratories, CA. All other materials were obtained from Sigma.

2.2. Cell line and cell culture

The human leukemia cells HL-60 were maintained in RPMI medium 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. The cells were split at the third day and were diluted 1 day before each experiment. Cell densities in culture did not exceed 5×10^5 cells/ml.

2.3. Assessment of cell viability

Cells were seeded at a density of 2×10^5 cells/ml and incubated with esculetin at various concentrations (0, 10, 20, 50 and 100 μM) for 24 h and 48 h. Thereafter, the medium was changed and 3-(4,5-dimethylthiazol-zyl)-2,5-diphenyltetrazolium bromide (MTT; 0.1 mg/ml) was added for 4 h. The viable cell number is directly proportional to the production of formazan which, following solublization with isopropanol, was measured spectrophotometrically at 563 nm.

2.4. Analysis of morphological changes

Untreated or esculetin-treated (100 μM, 24 h) HL-60 cells were examined for morphological changes by inverted microscopy. To assay the nuclear morphology, cells were harvested, placed on a glass slide using cytospin, fixed with 95% ethanol for 1 h and stained with 0.1% hematoxylin.

2.5. DNA gel electrophoresis

Untreated or esculetin-treated (100 μM, 0–24 h) cells were collected by centrifugation ($200 \times g$, 10 min), washed in PBS (with 1 mM $ZnCl_2$), resuspended (5×10^6 cells/ml) in 0.5 ml lysis buffer (0.5% sodium dodecyl sulfate, 100 mM EDTA, 10 mM Tris and 200 mg/ml RNase A; pH = 8.0) and incubated at 37°C for 1 h. Proteinase K was added at a concentration of 0.5 mg/ml and the cells were incubated at 50°C for 12 h. DNA was extracted with phenol, then ethanol was added and the mixture was left overnight at −80°C. After quantitative analysis of the DNA content by spectrophotometry (260 nm), an equal amount of DNA (20 μg) was electrophoresed in horizontal agarose gels (1.5%) at 1.5 V/cm for 3 h. DNA in gels was visualized under UV light after staining with ethidium bromide (0.5 mg/ml).

2.6. Measurement of DNA fragmentation

The DNA fragmentation of apoptotic cells induced by esculetin was determined using a sandwich enzyme immunoassay (ELISA) kit. Briefly, HL-60 cells grown in flasks were labeled with 5-bromo-2′-deoxy-uridine (BrdU; 10 μmol/ml) for 18 h. After labeling, the cells were collected and resuspended at a density of 1×10^5 cells/ml and then incubated with esculetin (0–100 μM) for 24 h. After centrifugation ($250 \times$ g, 10 min), part of the nuclear DNA of the apoptotic cells was released into the supernatant. The BrdU-labeled DNA can be easily detected and quantified photometrically (at wavelength 450 nm) by enzyme-linked immunosorbent assays using a monoclonal antibody against BrdU.

2.7. Analysis of cellular DNA content

DNA content of esculetin-treated (100 μM, 0–24 h) HL-60 cells was determined using a Becton Dickinson Immunocytometer with the Cycle TEST™ PLUS DNA reagent kit. Briefly, the cell pellets (5×10^5 cells) were suspended in 0.25 ml solution A (trypsin buffer) and

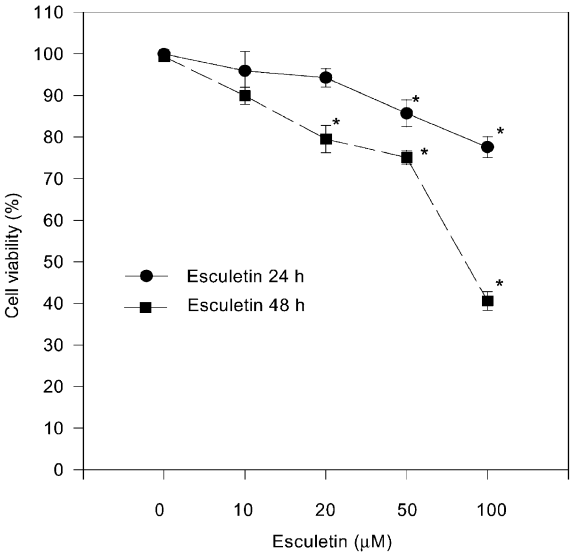

Fig. 2. Effect of esculetin on HL-60 cell proliferation. Cells were treated as described in the text. The results are presented as means ± S.D. of three independent experiments. * $P < 0.05$, compared with control group (0.1% DMSO).

incubated at 25°C for 10 min, then 200 μl solution B (trypsin inhibitors) was added and the mixture was incubated at 25°C for 10 min. Then 200 μl solution C (propidium iodide) was added and the mixture was incubated at 4°C for 10 min. The suspension was filtered through a 50-μm nylon mesh, and the DNA content of stained nuclei was analyzed by flow cytometry (Becton Dickinson). The distribution of DNA content was expressed as G_1, S, and G_2/M phase, inclusively. In addition, the percentage of

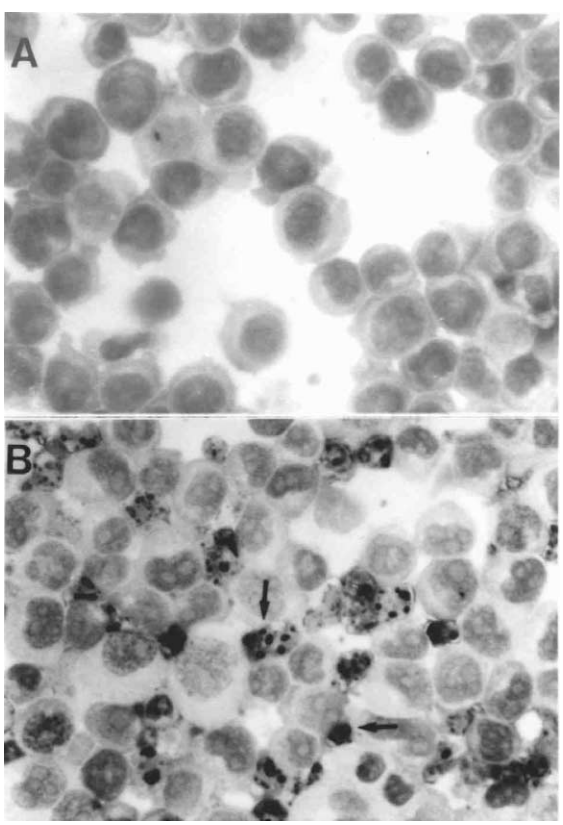

Fig. 3. Microscopic appearance of hematoxylin-stained nuclei of esculetin-treated HL-60 cells. HL-60 cells were (A) untreated (solvent control) or (B) treated with 100 μM esculetin for 24 h, then stained with hematoxylin and examined under a microscope (×400). Arrow indicates the condensed and fragmented nuclei.

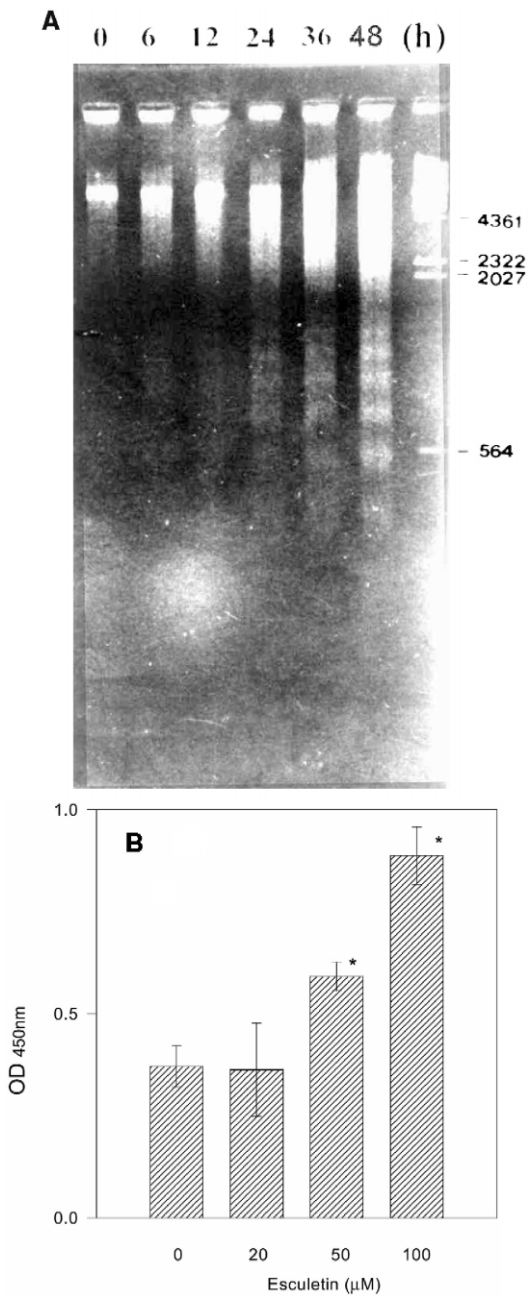

Fig. 4. Apoptotic phenomena induced by esculetin. (A) Agarose gel electrophoresis of DNA isolated from HL-60 cells treated with 100 μM esculetin for indicated times. After extraction, DNA samples (20 μg) were separated on a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV illumination. (B) DNA fragmentation induced by 100 μM esculetin for 24 h was determined photometrically (450 nm) with an ELISA kit. The results represent means ± S.D. of three independent experiments. * $P < 0.05$, compared with control group.

hypodiploid cells (pre G_1), which appeared in the cell distribution with DNA content less than G_1, was measured.

2.8. Preparation of subcellular fractions

The basic methodology for the preparation of mitochondria and cytosolic fractions was described by Tang et al. (1998). Various cells (3×10^6) at the end of the treatment were harvested and washed with ice-cold PBS. Cells were resuspended in 500 μl of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM leupeptin, 1 μg/ml pepstatin A and 1 μg/ml chymostatin). The cells were homogenized in the same buffer, with a Pyrex glass homogenizer using a type B pestle (40 strokes) and then centrifuged at $1000 \times g$ for 10 min 4°C. The resulting supernatant was subjected to $10{,}000 \times g$ centrifugation at 4°C for 20 min. The pellet (i.e., mitochondria) was first washed with the above buffer A containing sucrose and then solubilized in 50 μl of TNC buffer (10 mM Tris-acetate, pH = 8, 0.5% NP-40, 5 mM $CaCl_2$). The supernatant was recentrifuged at $100{,}000 \times g$ (4°C for 1 h) to generate cytosol. The protein concentration was determined with the Bio-Rad protein assay kit, and 25 μg of each fraction was loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis (SDS-PAGE). Protein was then blotted onto nitrocellulose (NC) membranes (Sartorious), and the membranes were reacted with primary antibodies (anti-cytochrome *c* from Transduction Laboratories; anti-α-tubulin from Sigma as internal control). The secondary antibody was a peroxidase-conjugated goat antimouse antibody. After binding, the bands were revealed with a commercial ECL kit.

2.9. Preparation of total cell extracts and immunoblot analysis

Cells were plated onto 15-cm^2 dishes at a density of 2×10^5 cells/ml with or without esculetin (100 μM,

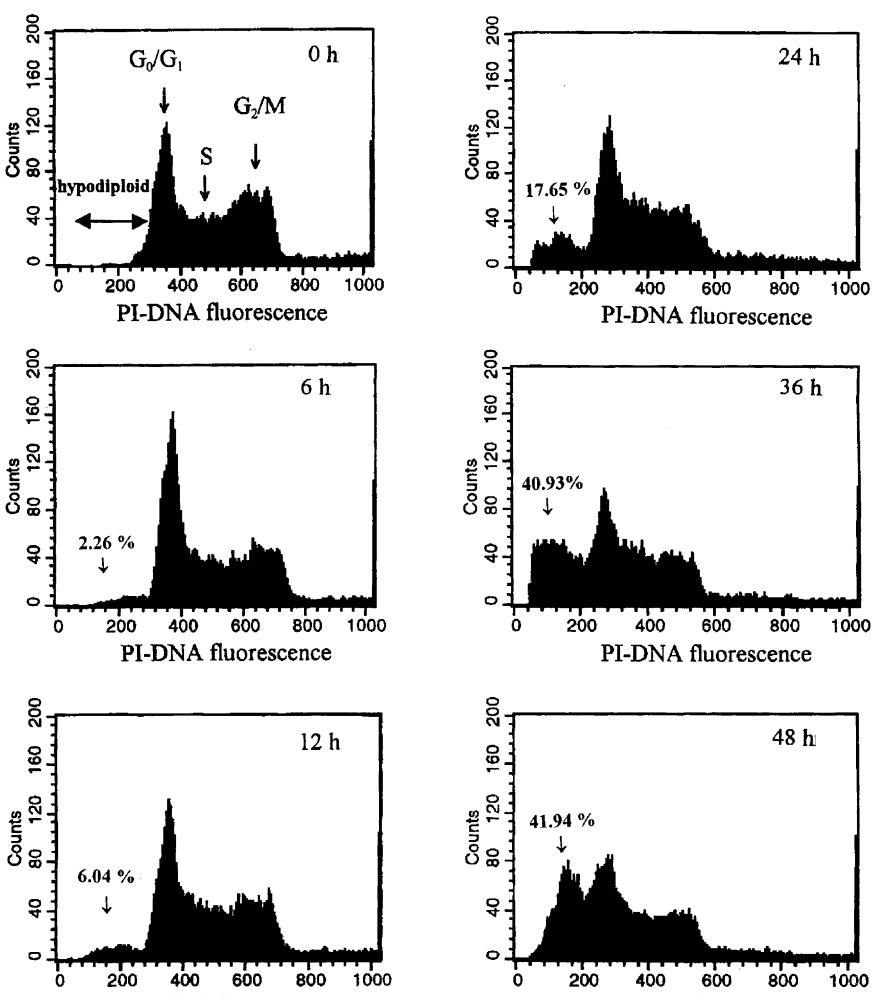

Fig. 5. Effect of esculetin on DNA content of HL-60 cells. Cells were exposed to 100μM esculetin for the indicated times, then washed and harvested. The cells were fixed and stained with propidium iodide, and the DNA content was analyzed by flow cytometry (FACS). The number of hypodiploid cells (pre-G1 phase) is expressed as a percentage of the total number of cells.

0–36 h) and harvested. To prepare the whole-cell extract, cells were washed with PBS plus zinc ions (1 mM) and suspended in a lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% NP 40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/ml aprotinin, 170 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride (pH = 7.5). After 30 min of rocking at 4°C, the mixtures were centrifuged (10,000 × *g*) for 10 min, and the supernatants were collected as the whole-cell extracts. The protein content was determined with the Bio-Rad protein assay reagent using bovine serum albumin as a standard, and an equal amount of total cell lysate was resolved on 10–12% SDS-PAGE gels along with prestained protein molecular weight standards (Bio-Rad). Protein was then blotted onto NC membranes (Sartorious), and the membranes were reacted with primary antibodies (anti-Bcl 2, anti-CPP32 and anti-PARP from Transduction Laboratories; and anti-α-tubulin from Sigma as internal control). The secondary antibody was a peroxidase-conjugated goat antimouse antibody. After binding, the bands were revealed with a commercial ECL kit.

2.10. Statistical analysis

Data are reported as means ± standard deviation of three independent experiments and evaluated by one-way analysis of variance (ANOVA). Significant differences were established at $P < 0.05$.

3. Results

3.1. Cytotoxicity of esculetin on HL-60 cells

Cell viability was assayed in cultures exposed to 0–100 μM esculetin for 24 and 48 h, respectively. Esculetin showed a concentration-dependent inhibitory effect on the growth of HL-60 cells (Fig. 2). Fifty and 100 μM of esculetin reduced the growth of HL-60 cells distinctly at 24 and 48 h. The cells had an abnormal appearance, with cell shrinkage, membrane blebbing and apoptotic bodies being detected by inverted microscopy (data not shown).

3.2. Induction of apoptosis by esculetin

Cells treated with 100 μM esculetin for 24 h showed typical apoptotic features: chromatin condensation (deeply dyed) and nuclear fragmentation (as evidenced by hematoxylin-stained nuclei; Fig. 3 indicated by arrow). In addition, after 100 μM esculetin treatment for 24 h, cells demonstrated a ladder-like pattern of DNA fragments, consisting of multiples of approximately 180–200 bp (Fig. 4A). Quantitative analysis of the DNA fragmentation induced by esculetin was performed using an ELISA kit with BrdU DNA labeling. A 24-h exposure of HL-60 cells to esculetin produced a concentration-dependent increase in the degradation of cellular DNA into small double-stranded fragments (Fig. 4B), being significantly higher than the

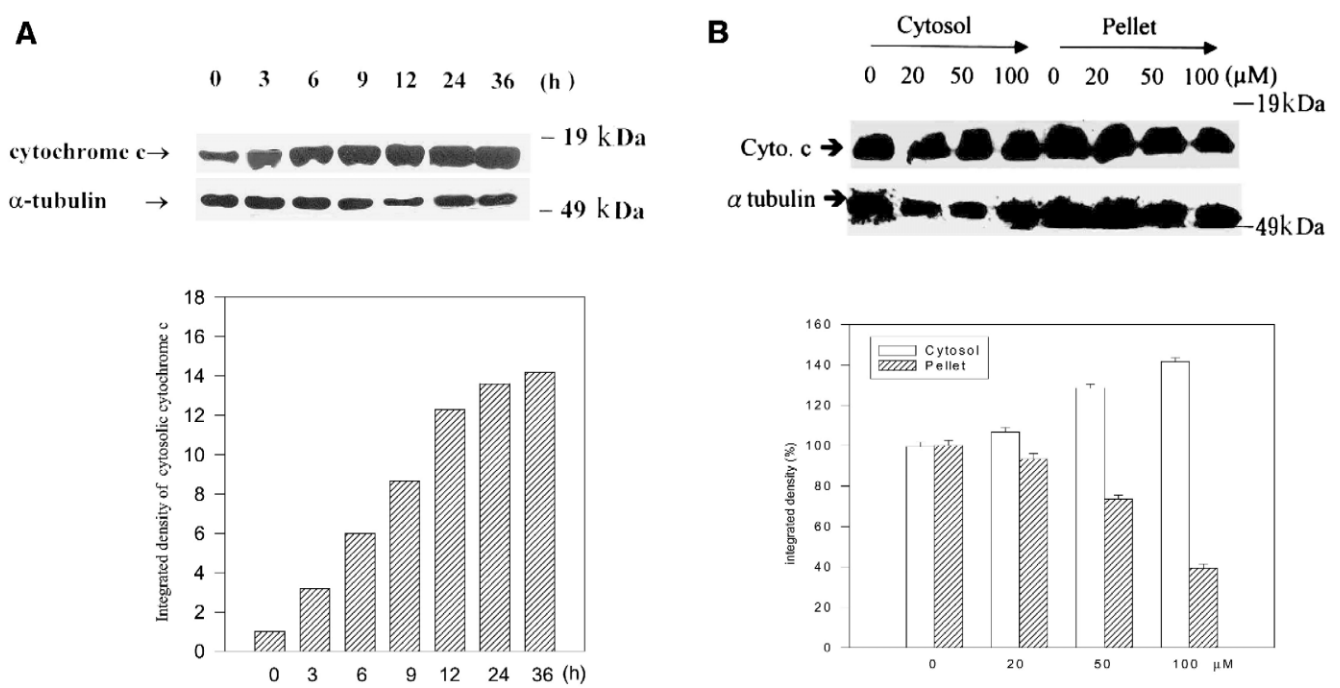

Fig. 6. Effect of esculetin on cytochrome *c* translocation (A) Equal amounts of protein from the cytosolic fraction of 3×10^6 HL-60 cells, which had been treated with 100 μM esculetin for the indicated times were analyzed by 15% SDS-PAGE and, subsequently, immunoblotted with antibody against cytochrome *c* and α-tubulin, which served as an internal control. (B) Equal amounts of protein from the cytosolic and pellet (mitochondria) fractions of 3×10^6 HL-60 cells, which had been treated with 0–100 μM esculetin for 12 h were analyzed by 15% SDS-PAGE and, subsequently, immunoblotted with the antibody against cytochrome *c* and α-tubulin, which served as an internal control. Densitometric quantitation (arbitrary units) of the autoradiogram was performed.

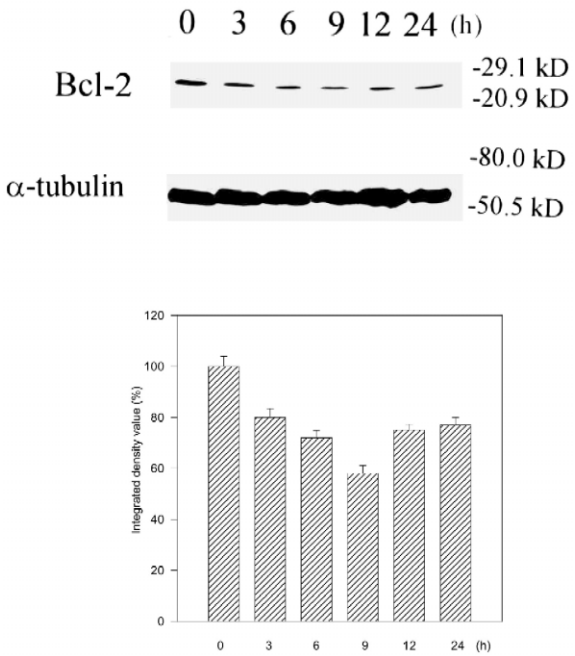

Fig. 7. Effect of esculetin on Bcl-2 protein of HL-60 cells. Equal amounts of protein from total cell lysates prepared from 8×10^6 HL-60 cells, which had been treated with 100 μM esculetin for indicated times were analyzed by 12% SDS-PAGE and, subsequently, immunoblotted with antibody against Bcl-2 and α-tublin as internal control. Densitometric quantitation (arbitrary units) of the autoradiogram was performed.

basal level at concentrations of 50 and 100 μM of esculetin. To further examine the effect of esculetin on apoptosis, we used flow cytometry to quantify the apoptotic state. We found that HL-60 cells exposed to 100 μM esculetin for 6, 12, 24, 36 and 48 h demonstrated 2.26%, 6.04%, 17.65%, 40.93% and 41.94% apoptosis (hypodiploid phase), respectively (Fig. 5).

3.3. Effect of esculetin on cytochrome c and Bcl-2 protein

Since cytochrome *c* is reported to be involved in the activation of the caspases that execute apoptosis, we examined the level of cytochrome *c* in the mitochondria and the cytosol by Western blot analysis. Results showed that the amount of cytosolic cytochrome *c* gradually increased from 3 h to 36 h (Fig. 6A). We also determined the concentration response effect of esculetin on cytochrome *c* translocation (Fig. 6B) after 12 h of treatment. Results revealed that esculetin increased the translocation of cytochrome *c* from the mitochondria to the cytosol.

Recently, investigations of the *bcl-2* gene family have shown a complex network regulating apoptosis. Bcl-2 is an integral membrane protein that prevents apoptosis in multiple biological systems (Yang et al., 1997). The expression of Bcl-2 correlates inversely with the susceptibility of cells to apoptosis. We examined the cellular levels of Bcl-2 after treatment of HL-60 cells with esculetin. Bcl-2 was decreased to 80% after a 3-h treatment and, subsequently, to 58% after a 9-h treatment, as compared with the level at 0 h (Fig. 7).

3.4. Effect of esculetin on CPP32 and PARP cleavage

CPP32 is a cytosolic protein that normally exists as a 32 kDa inactive precursor. It is cleaved proteolytically into a 20 and 10 kDa heterodimer when the cell undergoes apoptosis (Nicholson et al., 1995). In this study, activation

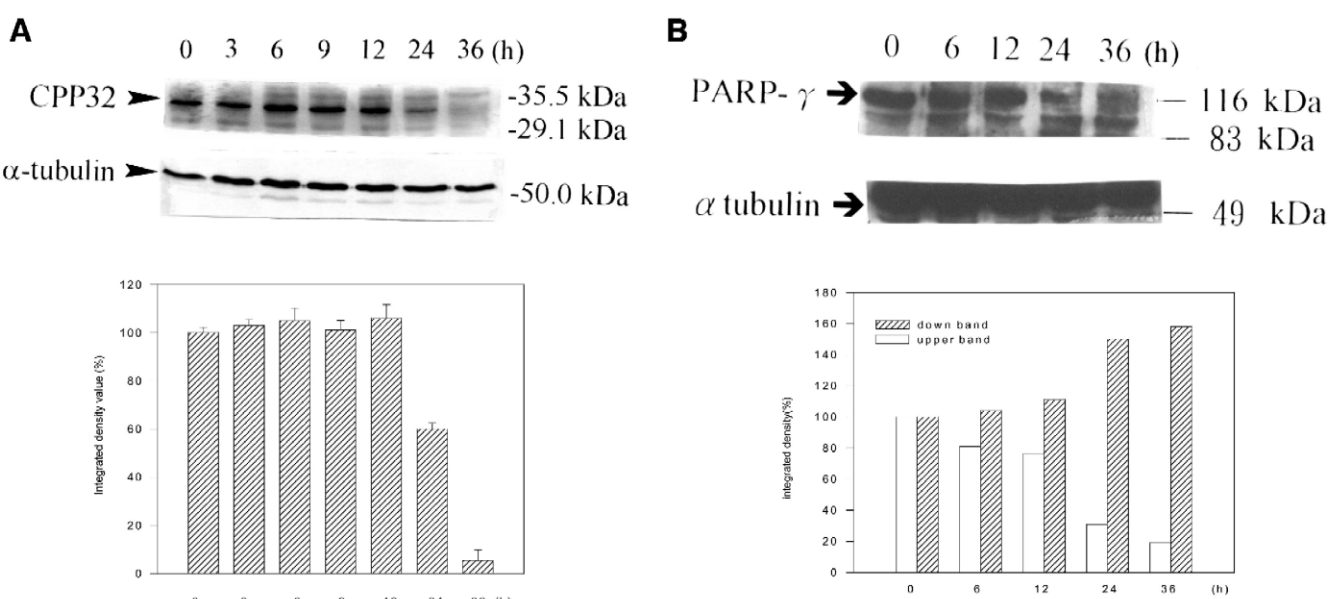

Fig. 8. Effect of esculetin on CPP32 and PARP. Total cell lysates (50 μg) of HL-60 cells treated with 100 μM esculetin for the indicated times were analyzed by 12% SDS-PAGE for CPP32 or 10% for PARP and, subsequently, immunoblotted with antibody against CPP32 or PARP and α-tubulin, which served as internal control. Densitometric quantitation (arbitrary units) of the autoradiogram was performed.

of CPP32 in response to esculetin treatment was assessed by the decline in the 32 kDa band. We observed that the inactive CPP32 band was apparently decreased after 24 h of esculetin treatment (Fig. 8A).

One of the substrates for caspase protease during apoptosis is PARP, an enzyme that appears to be involved in DNA repair and genome surveillance and integrity, in response to environmental stress. Proteolytic cleavage of PARP results in a characteristic shift of the protein upon electrophoresis from 116 to 89 kDa (Tewari et al., 1995). PARP cleavage was used as an indicator of caspase activation in response to esculetin treatment. It is obvious that PARP was cleaved after 24 h esculetin treatment (Fig. 8B). These results were consistent with the results of DNA gel electrophoresis, which revealed DNA fragmentation after a 24-h treatment with esculetin.

4. Discussion

Scientific interest in phenolic compounds has been provoked recently due to their anti-inflammatory, antimutagenic and anticarcinogenic properties (Huang and Ferraro, 1992; Newmark, 1992). Esculetin, a phenolic compound (Fig. 1), has been reported to possess antitumor (Nakadate et al., 1984), anti-inflammatory (Tubaro et al., 1988) and antioxidant (Paya et al., 1992) activities. It also inhibits the proliferation of breast cancer cells, possibly by its modulation of arachidonic acid metabolism (Noguchi et al., 1995; Hofmanova et al., 1996). Recently, esculetin has been demonstrated to be an efficacious agent in inhibiting the carcinogenic action of various chemicals, such as *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis in rats (Matsunaga et al., 1998) and benzo[a]pyrene plus 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in A/J mice (Hecht et al., 1999). In this paper, we noted that esculetin had a cytotoxic effect by inducing apoptosis in HL-60 human leukemic cells and that this effect was mainly associated with the release of cytochrome *c* from mitochondria.

Evidence to date has suggested that cytochrome *c*, which is usually present in the mitochondrial intermembrane space, is released into the cytosol following the induction of apoptosis by many different stimuli including CD95, tumor necrosis factor and chemotherapeutic agents (Sun et al., 1999). It is also reported that the released cytochrome *c* could initiate the cleavage and activity of caspase-3, thus triggering the apoptotic program (Zou et al., 1997). In this study, esculetin initiated cytochrome *c* translocation from the mitochondria to the cytosol in a time-dependent and a concentration-dependent manner (Fig. 6). It has been suggested that Bcl-2 could prevent apoptosis by blocking cytochrome *c* release from mitochondria (Yang et al., 1997). Considerable attention has been focused on the proto-oncogene Bcl-2, which encodes an inner mitochondrial protein that reportedly antagonizes apoptosis in many tumor cells (Lotem and Sachs, 1993). Decreased expression of this protein might contribute to drug-mediated lethality. Recently, the induction of apoptosis in HL-60 cells by taxol (Bhalla et al., 1993; Ibrado et al., 1997), curcumin (Kou et al., 1996) and retinoid *N*-(4-hydroxyphenyl) retinamide (Reed and Pierotti, 1995) has been found to be temporally associated with the downregulation of Bcl-2. Consistent with this, we found that esculetin-induced apoptosis was associated with the downregulation of Bcl-2 after a 3-h treatment (Fig. 7).

Recently, attention has been focused on the possibility that intracellular proteases may play a critical role in the initiation of programmed cell death (apoptosis). Caspases are a family of cysteine proteases that are activated during programmed cell death (Kumar and Harvey, 1995; Nicholson et al., 1995). These proteases are synthesized as proenzymes, which are proteolytically cleaved into active heterodimers. Caspases can be grouped according to their substrate specificities, which are largely determined by the amino acids preceding the cleavage site aspartic residue (Talanian et al., 1997). The substrate specificity of one group of caspases that includes caspases-6,-8 and -9 is valine/leucine–glutamic acid–any amino acid-aspartic acid (V/LExD), a site similar to that found in caspase proenzyme. Therefore, these caspases may function as the initiators of a proteolytic cascade by activating pro-caspases to amplify a death signal. The substrate specificity of a second group, consisting of caspases-2,-3 and -7, is aspartic acid–glutamic acid–any amino acid-aspartic acid (DExD), a cleavage site similar to that found in many target proteins that are cleaved during apoptosis, which suggests that these caspases function during the effect phase of cell death. However, the caspase cascade is not yet well defined, and some caspases may serve both upstream and downstream functions. According to our results, cytosolic cytochrome *c* levels increased from 3 h onward, whereas, CPP32 was activated 24 h after esculetin treatment (Fig. 8). We suppose that CPP32 is a downstream caspase in esculetin-induced apoptosis. Other caspases involved in esculetin-induced apoptosis need further investigation.

In conclusion, esculetin exhibits an antiproliferative effect by inducing apoptosis that is associated with cytochrome *c* translocation and caspase activation in HL-60 cells. Thus, we suggest that the anti-tumor effect of esculetin is, at least in part, due to its apoptosis-inducing activity.

Acknowledgements

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